

HISTOCHEMICAL LOCALIZATION OF PHOSPHATASES IN THE STABLE FLY, *STOMOXYS CALCITRANS* (L.), USING NAPHTHOL AS-PHOSPHATE¹

SHAHID H. ASHRAFI² AND FRANK W. FISK³

Department of Zoology and Entomology, The Ohio State University, Columbus 10

INTRODUCTION

The detection of phosphatases in insects began about 20 years back when Nakamura (1940) localized alkaline phosphatase in the silk gland of *Bombyx*. Later Krugalis (1945) demonstrated alkaline phosphatase in the chromosomes of the salivary glands of *Drosophila melanogaster* larvae, and Bradfield (1946) reported the presence of the same enzyme in insect cells which synthesize fibrous proteins. Day (1949), using Gomori's technique, studied the distribution of alkaline phosphatase in insect tissues. Yao (1950a, b) demonstrated phosphatases in various tissues of *Drosophila melanogaster* during embryonic and postembryonic development. Belden (1958), using Gomori's method, made a detailed study of histochemical localization of acid and alkaline phosphatases in adult males of DDT-resistant and DDT-susceptible strains of the house fly, *Musca domestica*. Salkeld (1959), using naphthol AS-acetate as a substrate found a pronounced nonspecific esterase activity in the posterior lobe of salivary glands and the fore part of the midgut of the large milkweed bug, *Oncopeltus fasciatus*, Dall. Recently, Burstone (1958c) reported a new technique, in which naphthol AS-phosphates were used to get precise localization of phosphatases in mammalian tissues. He stated that this technique has certain advantages over other histochemical methods in that: (a) naphthol AS-phosphates are among the most substantive and insoluble naphthols; (b) diazonium salts couple rapidly with enzymatically released free naphthol from the substrate and form a dye product of homogeneous (non-crystalline) structure; and (c) the dye-stuff produced is very insoluble and remains at the site of its formation. In view of the advantages of Burstone's technique, it was selected for the present investigation of acid and alkaline phosphatases in the stable fly, *Stomoxys calcitrans* (L.).

MATERIALS AND METHODS

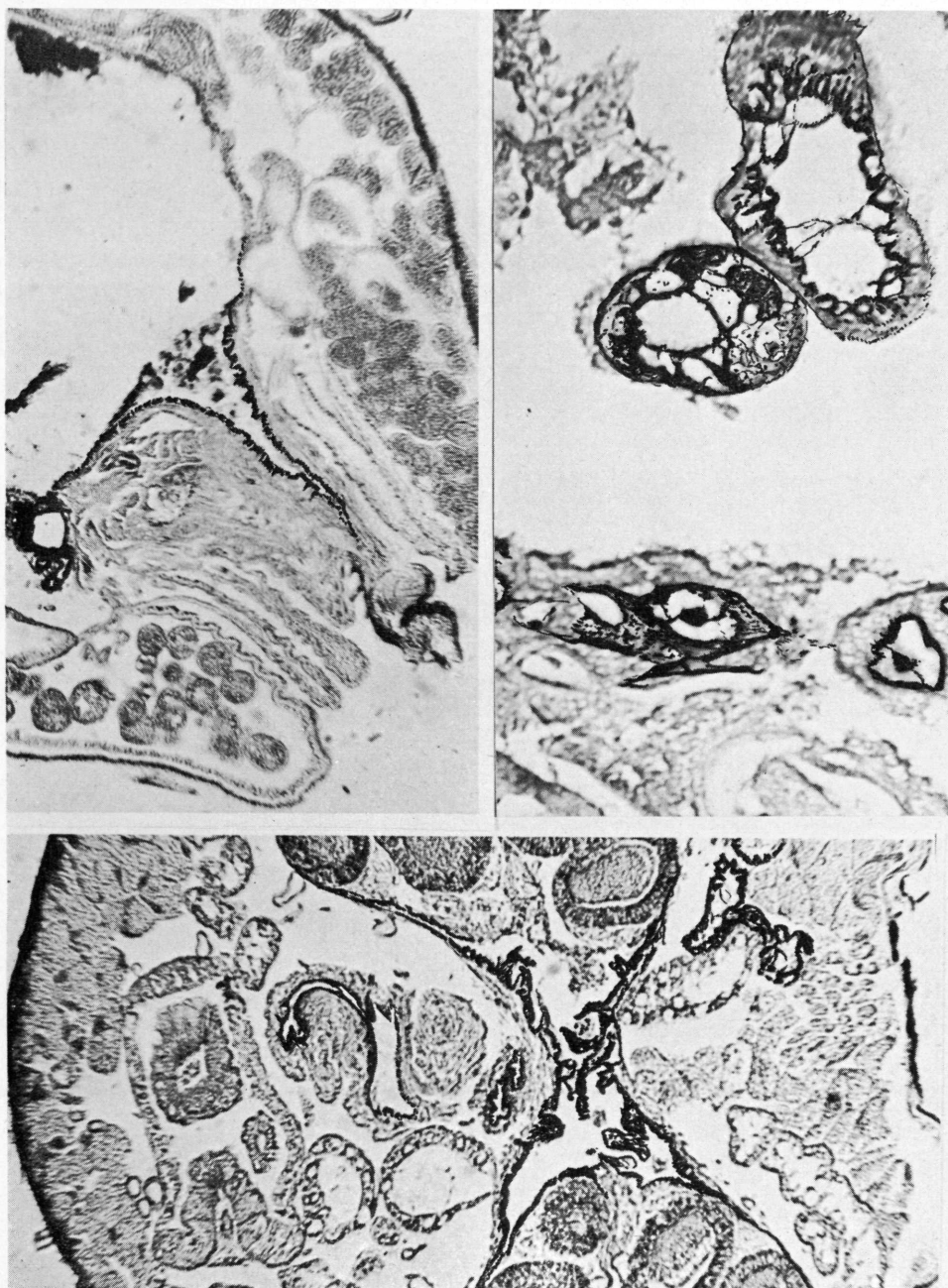
Live three-day-old adult stable flies were chilled for 30 minutes at a temperature of -15°C to avoid serious postmortem distortion of phosphatases (Gomori, 1952). Next, the tissues were fixed and dehydrated in a mixture of equal volumes of cold acetone and absolute alcohol for twelve hours. They were transferred to fresh fixative at 12, 24, and 36 hours. After 48 hours, the tissues were washed with cold chloroform. Ether was avoided because it was found to interfere with sharp localization of phosphatases in stable fly tissues. The embedding was done in paraffin wax under reduced pressure and the tissue sections were cut at 9 microns.

The sections were dewaxed in cold xylene for 30 minutes and were then hydrated by passing them successively through alcohols of 100, 90, 75, and 35 percent. The sections were exposed for two minutes in each grade of alcohol. Finally, sections were immersed in distilled water for one minute, then transferred to the incubation mixture and held at a water bath temperature of 38°C for 30 minutes to two hours.

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²Present address: Entomology Section, Pakistan Council of Scientific and Industrial Research, 35 P.N.H. Lines, Karachi 4, Pakistan.

³Associate Professor, Department of Zoology and Entomology.



- FIGURE 1. (Upper left) Longitudinal section of hindgut. Rectal contents and the epithelium of the posterior part of the rectum showing acid phosphatase activity.
- FIGURE 2. (Upper right) Cross section of Malpighian tubules, showing localization of acid phosphatase in the protoplasm of the cell.
- FIGURE 3. (Bottom) Cross section of female stable fly abdomen. Acid phosphatase activity is present in the cytoplasm and nuclei of the epithelial cells of oviduct and uterine glands.

The incubation mixtures were prepared as follows (Burstone, 1958c):

Five mg of substrate (Naphthol AS-BI phosphate for acid phosphatase, and naphthol AS-MX phosphate for alkaline phosphatase) were dissolved in 0.25 ml N, N-dimethylformamide followed by 25 ml distilled water and 25 ml of buffer (0.2 M acetate of pH 5.2 for acid phosphatase, and 0.2 M tris-hydroxymethylamino-methane buffer of pH 8.3 for alkaline phosphatase). Then 30 mg of Red Violet LB salt for acid phosphatase or of Fast Blue BBN salt for alkaline phosphatase was added. This mixture was shaken and filtered into a Coplin jar. Two drops of 10 percent $MnCl_2$ were added to the incubation mixture prepared for acid phosphatase localization.

As a result of phosphatase activity during incubation, AS-naphthol was liberated from naphthol AS-BI phosphate and naphthol AS-MX phosphate. The liberated AS-naphthol couples immediately with diazonium Red Violet LB salt, forming an insoluble red pigment in the case of acid phosphatase or with diazonium Fast Blue BBN salt, forming a blue pigment in the case of alkaline phosphatase.

After incubation, the sections showing the acid phosphatase activity were counterstained with hematoxylin and those showing alkaline phosphatase with eosin. The sections were mounted in glycerol jelly.

RESULTS

Acid and alkaline phosphatases appeared to be widely distributed in the cytoplasm and nuclei of the cells of the tissue in which they were localized. The acid phosphatase activity of the nucleus was dominant over the acid phosphatase activity in the cytoplasm, while the reverse held for alkaline phosphatase. In some tissues both acid and alkaline phosphatases were shown to be present at the same sites.

Acid phosphatase activity.—Only moderate phosphatase activity was found in the epithelium of the posterior part of the rectum (fig. 1). The distal part of the salivary gland was also a site of moderate activity. This activity was oriented in the luminal margin of the epithelial cells of the salivary gland, and was decreasing towards the proximal part of the gland.

A distinct localization of acid phosphatase was observed in the luminal brush border of the malpighian tubules (fig. 2). The activity was noted in both distal and proximal parts of the tubules.

Acid phosphatase was not found in the ovarian follicles of three-day-old female flies, but was widely distributed in the cytoplasm and nuclei of the epithelial cells of the oviduct and uterine glands (fig. 3). The testes were devoid of acid phosphatase activity, but testicular ducts and seminal vesicles did show activity.

Alakline phosphatase activity.—The entire midgut epithelium showed maximum activity, with the brush border of the mesenteron exhibiting a dense localization of alkaline phosphatase activity (fig. 4). A trace of alkaline phosphatase activity was noted around the base of the rectal papillae and intimal lining of the posterior part of the rectum.

Moderate activity of alkaline enzyme was observed in the proximal part of the salivary glands. The distal lobe of each salivary gland showed a strong intracellular orientation of the enzyme (fig. 5). Most of the activity was found in the lumen of the gland and the cells bordering it.

A strong alkaline phosphatase activity was noted in the brush border of the distal part of the malpighian tubules. The cytoplasm was the seat of a sharp alkaline phosphatase activity in the cells of malpighian tubules.

Marked alkaline enzyme activity was found in the cytoplasm and nuclei of the oviduct and uterine gland epithelial cells (fig. 6). Testicular ducts showed traces of alkaline phosphatase activity.

DISCUSSION

Several authors have mentioned that histochemical localization of an enzyme

depends upon the initial fixation, substrate used, product of hydrolysis, and end-product of dye pattern formed at the site of enzyme activity. Danielli (1954) summarized the factors necessary to obtain good results as: (a) solubility of the precipitated end-product due to enzymatic activity should be low; (b) an

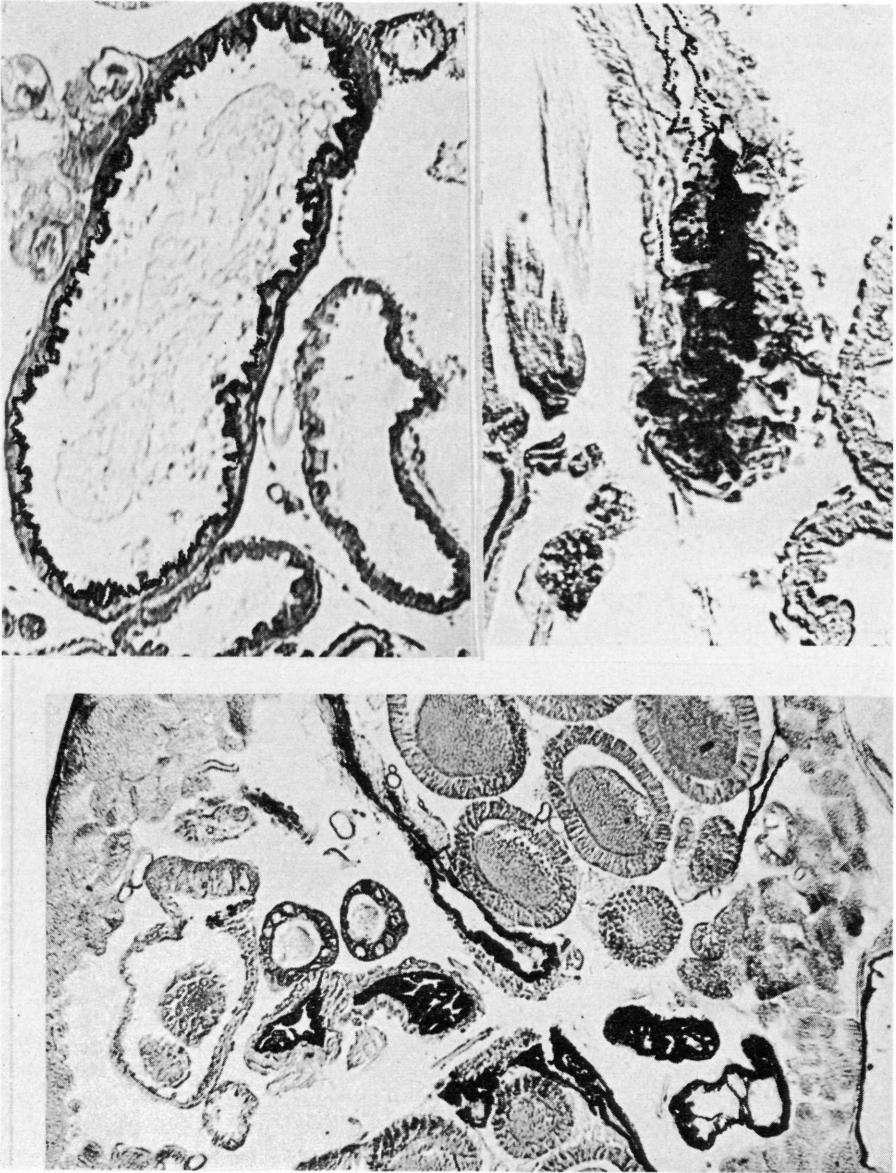


FIGURE 4. (Upper left) Longitudinal section of midgut, showing alkaline phosphatase activity in the midgut epithelium.

FIGURE 5. (Upper right) Longitudinal section of salivary gland. Most of the alkaline phosphatase activity is confined to the lumen and the cells adjacent to it.

FIGURE 6. (Bottom) Cross section of a female stable fly abdomen. Alkaline phosphatase activity is present in the cytoplasm and nuclei of the epithelial cells of oviduct and uterine glands.

abundance of precipitation nuclei (crystallization nuclei) should be present; and (c) the rate of enzymatic action should be sufficient to ensure a considerable amount of released phosphate. The first factor was satisfied when the solubility of the precipitated end-product was found almost nil at pH 5.2 and pH 8.3. The second factor was satisfied by the findings that usually all sites of the cell had high concentrations of precipitation centers. Whether our results satisfy the third factor of Danielli may be questioned, because many authors reported 65 percent loss of phosphatase activity during fixation, dehydration and embedding. In the present investigation, acetone-fixed and paraffin-embedded tissues have been used, although it has been reported (Eränko, 1951; and Samorajski, 1960) that cold acetone fixation is destructive to acid phosphatase. Seligman (1951) reported the use of cold neutral formalin fixation to overcome this difficulty. He showed that with cold formalin fixation acid phosphatase activity was preserved somewhat better, but that alkaline phosphatase was greatly reduced. Since the biochemical tests had shown a high concentration of acid phosphatase and a low concentration of alkaline phosphatase (Ashrafi, 1960), the method of fixation selected was that which favored the alkaline phosphatase. Therefore, acetone-fixation and paraffin-embedding may limit the present azo-dye procedure due to the possibility of negative results at the site of weak enzyme activity. However, this procedure gave an accurate and sharp localization at the sites of strong enzyme activity. Therefore, Danielli's third factor was at least satisfied in the regions where sufficient enzyme activity remained in the sections after preparation.

Acid phosphatase activity was not detected in the stomodaeal region. Only moderate activity was noted in the posterior rectal part of the digestive system. A weak acid phosphatase activity was localized in the salivary glands of the stable fly. Prior to this work only Belden (1958) reported acid phosphatase activity in insect salivary glands. A distinct acid phosphatase activity was found in the stable fly malpighian tubules. Similar findings were reported by Drilhon and Busnel (1945), Yao (1950), and Belden (1958) in other insects. Testes of the three-day-old adult stable flies were devoid of acid phosphatase activity, but testicular ducts and seminal vesicles showed weak acid phosphatase activity. The activity was also localized in oviducts and uterine glands. Belden reported the presence of acid phosphatase activity in the testicular ducts of the house fly.

Alkaline phosphatase activity was localized in the entire midgut, and the fore-midgut showed the maximum activity. Waterhouse and Stay (1955) reported alkaline phosphatase activity in the anterior and posterior midgut of *Lucilia cuprina* (Wied.) larvae, and showed the storage of glycogen and lipids in the same region. Traces of alkaline phosphatase were also noted around the base of the rectal papillae and intimal lining of the posterior rectum of the stable fly. A strong alkaline phosphatase activity was noted in the distal part of the stable fly malpighian tubules. Similar observations were made by Day (1949) in *Lucilia cuprina*, Yao (1950) in *Drosophila melanogaster*, and Belden (1958) in *Musca domestica*. The sharp orientation of alkaline phosphatase was found in the lumen of the salivary gland and the cells bordering it. Krugalis (1945), Bradfield (1946), Day (1949), Yao (1950), and Belden (1958) reported strong alkaline phosphatase activity in the salivary glands of other insects. Testicular ducts, oviducts and uterine glands showed weak alkaline phosphatase activity. Similar results were reported by Day and Belden.

Acid and alkaline phosphatase activity was not detected in the tracheae, nerves, muscles, or fat bodies of the stable fly, while it was reported in certain of these tissues by Day, Yao, and Belden in the insects they studied.

SUMMARY

Acetone-fixed and paraffin-embedded sections of the adult stable fly tissues were incubated in a mixture of naphthol AS-phosphates along with diazonium salts.

Moderate acid phosphatase activity was localized in the epithelium of the posterior part of the rectum, distal part of the salivary glands, testicular ducts, and the seminal vesicles. Strong acid phosphatase activity was observed in the malpighian tubules, epithelial cells of the oviduct, and uterine glands.

Moderate alkaline phosphatase activity was shown around the base of rectal papillae, intimal lining of the posterior part of the rectum, proximal part of the salivary gland, and testicular ducts. Strong alkaline phosphatase activity was noted in the fore-midgut, distal lobes of the salivary glands, distal part of the malpighian tubules, oviducts and uterine glands. These findings were compared with the reports of others working with insect tissues.

Positive results with naphthol AS-phosphates in the presence of diazonium salts of higher coupling energy offer conclusive proof of acid and alkaline phosphatase activity at the site of dye deposition. Hence, the naphthol AS-phosphates technique of Burstone can be used to accurately localize phosphomonoesterases in insects.

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The figures were reproduced from photomicrographs made in color by The Ohio State University Department of Photography.

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